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Research Article

Performance of SNP markers for parentage analysis in the Italian Alpine brown bear using non-invasive samples

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Abstract

Determination of parentage provides valuable information for the conservation of wild populations, for instance, by allowing the monitoring of breeding success and inbreeding. Between 1999 and 2002, nine brown bears (*Ursus arctos*) were translocated to augment the remnant population of a few surviving individuals in the Italian Alps, but only part of them reproduced, with a higher inbreeding risk occurrence in the long-time. Currently, in the Alpine population, parentage tests are assessed through the analysis of 15 microsatellite loci (STRs), but the reduction of genetic variability in future generations will need the use of additional informative markers. Single nucleotide polymorphisms (SNPs) have been proven to be useful and reliable in individual identification and family reconstruction; moreover, they can perform well on low-quality samples. In this study, we analysed 51 SNPs to generate a SNP multilocus genotype dataset of 54 Alpine brown bears (*Ursus arctos*) and compared its performance in parentage analysis with the validated STR dataset. We found that SNPs alone are not sufficient to determine parentage relationships, but the combination of SNPs and STRs provided unambiguous parentage assignments. The combined panel also performed better than STRs when true parents were not present in the dataset and, consequently, showed higher values of assignment probabilities.

Key words: Colony, FRANz, markers combination, microsatellites, monitoring, *Ursus arctos arctos*

Introduction

Parentage determination may greatly aid the management of wild populations of conservation concern (Jones and Ardren 2003; Hauser et al. 2011) since it provides information about reproductive success and inbreeding (Wilson et al. 2002; Vonholdt et al. 2008; De Barba et al. 2010a; Stenglein et al. 2011). Such informa-

tion can also contribute to answering ecological questions related to the mating system, social organisation and dispersal behaviour (Webster and Reichart 2005; Moore et al. 2014) and can be used in reintroduction programmes to optimise translocation strategies for endangered species (Labuschagne et al. 2015; Wright et al. 2015). Molecular markers are useful for inferring parentage in wild populations when it is difficult to collect information from field observations (Blouin 2003; Pemberton 2008). Moreover, non-invasive genetic methods offer a great opportunity to infer parentage in wild and elusive populations without disturbing animals in their habitats (Taberlet et al. 1999), although DNA from biological traces is often degraded and scarce (Navidi et al. 1992; Broquet and Petit 2004).

Microsatellite loci, also known as short tandem repeats (STRs), are multi-allelic and highly polymorphic markers that have been routinely used in the last decade for parentage analysis in wild populations, also by starting from non-invasive samples (Constable et al. 2001; Nielsen et al. 2001; De Barba et al. 2010a; Caniglia et al. 2014). However, STRs are prone to genotyping errors with low or degraded DNA (Dewoody et al. 2006) and can produce false genotypes or incorrect parentage assignments (Pompanon et al. 2005). Moreover, recent simulation studies indicate that STRs provide less precision for relatedness, particularly in small populations where allelic diversity may be low (< 4 alleles per locus: Robinson et al. (2013); Taylor (2015)).

Single nucleotide polymorphisms (SNPs) are another type of marker of increasing popularity for many conservation genetic studies. They are polymorphic sites dispersed into the genome; differently from STRs, they can be easily scored, record high genotyping success and low error rates (Morin et al. 2004; Anderson and Garza 2006). Moreover, SNP data do not require allele calibration amongst different laboratories, thus genotypes can be easily compared when transboundary individuals move across different areas monitored by different research groups (Vignal et al. 2002; Pompanon et al. 2005). SNPs differ from STRs as they are bi-allelic and, therefore, offer less information on a marker-by-marker basis (Marth et al. 2001; Glaubitz et al. 2003); nevertheless, a high number of SNPs is easily obtained, thereby compensating for the low per-SNP information content.

Furthermore, the recently-emerged microfluidic genotyping platforms have shown very low copy number detection thresholds and are, thus, particularly suitable for the amplification of poor-quality DNA (von Thaden et al. 2020). SNPs have proven to be effective in several parentage studies of cattle and wild populations of fish, mammals and birds (see Flanagan and Jones 2019 for a recent compendium of 58 SNP-based parentage analyses published), even for populations that face low genetic diversity (Tokarska et al. 2009; Wright et al. 2015; Campbell et al. 2019; Galla et al. 2020). Several studies compared the efficiency of STRs and SNPs in parentage testing (Labuschagne et al. 2015; Weinman et al. 2015; Kaiser et al. 2017). SNP analysis is relatively easy to carry out, but finding and validating new SNP markers is still costly. Thus, it may be simpler to test pre-existing SNPs than to isolate new ones; sharing markers would be also a chance to allow a comparison of the genetic variability between populations. Recently, an increased number of empirical studies have demonstrated the utility of SNP markers in kinship analysis also by starting from non-invasive samples (Kleinman-Ruiz et al. 2017; Kraus et al. 2015; see also Carroll et al. (2018) and Ekblom et al. (2021)), thus enabling their use in the study of wild populations with no disturbance to individuals.

The Italian Alpine bear population (*Ursus arctos*) lives in two separate areas: the first subpopulation inhabits the central Italian Alps, while the second, in eastern Italy, constitutes the expansion front of the Dinaric Mountain population (Fig. 1). A geographic and numeric contraction occurred between the 18th and 20th centuries in the Alps, because of human persecution, habitat loss and fragmentation. By the 1900s, only a few individuals survived (≈ 3) in the Trentino Region (Kohn et al. 1995) and the population was considered biologically extinct (Mustoni et al. 2003). The translocation of nine bears from Slovenia, which took place during the 2000s, was the origin of the central Alpine bear population that is presently increasing both in numbers and distribution (De Barba et al. 2010a; Tosi et al. 2015). All released bears were VHF - radio collared and the reconstituted population has been intensively monitored since the first release till now. For management reasons, additional trapping was carried out from 2000 to 2014; 12 more individuals born in the area were fitted with GPS-collars and monitored through telemetry. VHF-/GPS-tracking and the possibility to combine fingerprinting analysis and camera trap data to determine litter size of reproductive females enabled us to keep good track of population development, individual fates and cub production, especially for founders and the first generation of new-borns, which had a narrow distribution area. The latest estimates, based on genetic Capture–Mark–Recapture from opportunistic and systematic sampling, approximate the population size to 73–92 bears in 2021 (Groff et al. 2019). Additionally, although males from the Dinaric population are regularly observed in the eastern Italian Alps, there are no cases of proven reproduction, thus gene flow to the reintroduced population is not known to occur (Krofel et al. 2010; Skrbinsek et al. 2012).

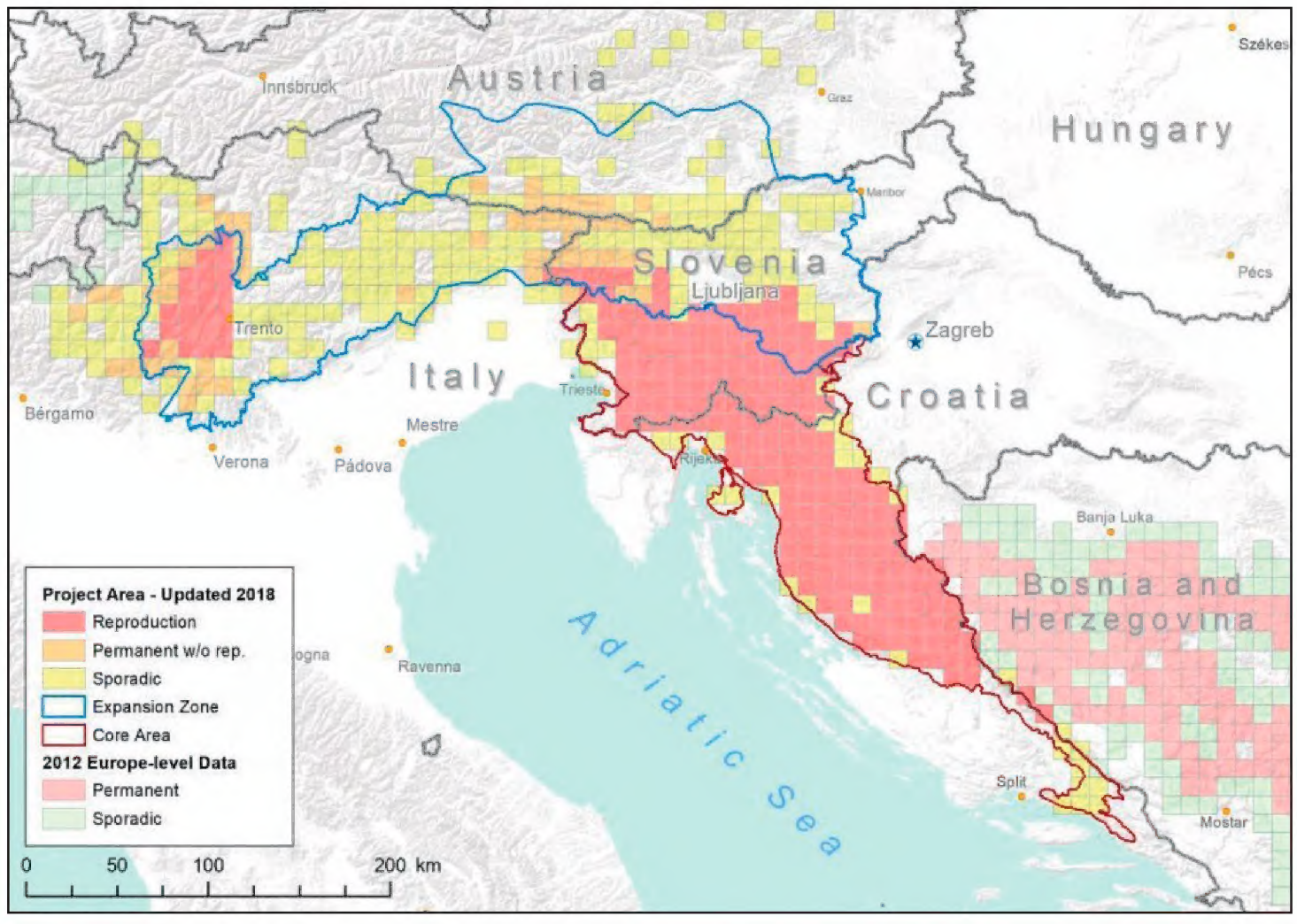

Figure 1. Brown bear distribution in the Italian Alps and neighbouring countries (from Skrbinšek et al. (2018)). Permanent presence, reproduction (red squares) – areas where cubs were confirmed within the last three years; permanent presence without reproduction (orange squares) – areas where bears have been present for at least three years over the last five years; sporadic presence (yellow squares) – areas where bear presence has been documented for fewer than three seasons in the last five years' period.

Although the status of the brown bear is categorised as “least concern” in its worldwide distribution area (McLellan et al. 2016), it is critically endangered in the Italian Alps (Stoch and Genovesi 2016) due to the limited number of individuals and geographic isolation. Therefore, it is essential to be able to study reproductive success and inbreeding levels to ensure the persistence of this population over time. Between 2000 and 2014, approximately 6000 non-invasive samples have been collected in the Alpine area to monitor the demographic trend of the reintroduced population. So far, 15 STR loci and two different regions for molecular sexing have been used for individual identification and parentage analysis (Probability of Identity PID = 3.7×10^{-13} and Probability of Identity between siblings PIDsibs = 6.6×10^{-06}; Allelic Drop-Out rates ADO = 0.041–0.168; De Barba et al. (2010a); Davoli et al. (2018); Giangregorio (2018)). Details on the genetic monitoring programme of the bear population in the Italian Alps during the reintroduction time frame are available in Suppl. material 1: tables S1, S2.

Despite the fact that the robustness of the STR protocol has been providing helpful information in the kinship analysis (Suppl. material 1: table S2), there are still critical issues in the peak scoring and the genotyping success due to fragment length, both conditions that increase the probability of genotyping error. Moreover, fluctuation in capillary electrophoresis makes difficult the recognition of the allele scoring when a reference genotype, previously analysed, is lacking.

Recently, a 96 × 96 SNP-chip comprising 85 autosomal SNPs, seven sex chromosome markers and four mtDNA markers was developed by Norman et al. (2013) and Norman and Spong (2015) and is currently used for bear monitoring in Scandinavia (J. Kindberg, pers. comm.). Amongst those, 51 autosomal and six sex chromosome SNP markers resulted in being variable also in the Alpine populations (Giangregorio et al. 2018) and were selected to be tested for individual and sex identification.

Here, we evaluate: i) the performance of the 51 SNPs in parentage analysis using non-invasive hair samples and ii) their reliability as compared to 15 STRs utilised in the monitoring of Alpine brown bear (Giangregorio 2018, Suppl. material 1: table S2) and a case study (Davoli et al. 2018). Given the intense monitoring programme, this population offers a great opportunity to test the suitability of SNP-based parentage analysis through non-invasive samples because the independent STR-based results and field data can be compared to SNP-based parental assignments.

Methods

Sampling methods

Hairs were collected in the Italian area of bear presence (Fig. 1) between 2000 and 2014, with both systematic and opportunistic methods through barbed-wire traps, transects and rub trees, during normal activities of various agency personnel and by volunteers or following notification by third parties, for example, after a damage event. The sampling activities were carried out for the annual monitoring of the species, coordinated by regional authorities and the Italian Institute for Environmental Protection and Research (ISPRA). Annual reports on

bear presence are regularly published online (https://grandicarnivori.provincia.tn.it/Large-Carnivores-Report).

Sampling procedures followed the guidelines provided by the interregional action plan for the conservation of brown bears in the Italian Alps (PACOBACE) (AA.VV. 2010). Samples were collected using sterilised forceps or latex gloves and placed in envelopes, then stored in silica desiccant or alternatively in 95% ethanol (De Barba et al. 2010b).

STR genotypes, databank and sample selection

STR genotyping using methods developed by De Barba et al. (2010a) and implemented by Davoli et al. (2018), was carried out during the entire non-invasive monitoring project on the Alpine brown bear and made it possible to detect the asynchronous presence of 85 bears between 2000 and 2014 (De Barba et al. 2010a; Groff et al. 2016; Giangregorio 2018). For evaluating the SNPs panel, a total of 71 hair samples belonging to 71 different bears, genotyped and stored at -20 °C at ISPRA, were extracted using the Qiagen DNeasy Blood & Tissue Kit (Qiagen inc., Hilden, Germany) following the manufacturer's instructions. DNA or biological material of the remaining 14 bears was no longer available and they were not included in this comparative study. The samples of three bears from the Dinaric population were also added to test parentage analysis; thus a total of 74 genotypes, corresponding to the same number of individuals, was utilised in this study.

SNP genotyping

DNA aliquots (n = 74) were sent to the Swedish University of Agricultural Sciences (SLU), to be amplified on the Biomark platform (Fluidigm Corporation, San Francisco, USA) with the 96 × 96 SNP panel developed by Norman et al. (2013) and colleagues. Clusters obtained from SNP genotyping were visualised in the Fluidigm SNP Genotyping Analysis software v.3.1.2. We applied the same filtering procedures for SNP validation and sex identification identified in Giangregorio et al. (2018), who found 51 informative SNPs in the Alpine brown bear population: i) we removed SNPs which gave no amplification signal in any sample and those which were monomorphic, excluding Y-chromosome and mitochondrial SNPs, which are always haplotypic; ii) to prevent possible errors in genotypes, we removed all loci that showed unclear cluster affiliation or unusual clustering patterns and iii) those which showed a departure from Hardy Weinberg equilibrium. Only SNPs which passed the screening were analysed. A flowchart showing the SNP genotyping steps and the filtering process is shown in Suppl. material 1: table S3.

We replicated a proportion of the samples (16 samples were replicated once, while 13 were replicated three times – see Suppl. material 1: table S4 for details) to calculate the percentage of positive amplifications and error rates with GIMLET v. 1.3.3 (Valière 2002), following the procedures described in Giangregorio et al. (2018). Afterwards, consensus genotypes amongst the replicates were created using a conservative approach where allele inconsistencies amongst replicates were not called and were marked as missing alleles. Consensus genotypes with a call rate ≤ 70% were discarded.

SNP and STR marker statistics

Deviations from the Hardy-Weinberg equilibrium (HWE) were computed using the exact test in Genepop (Raymond and Rousset 1995). SNPs that showed a departure from the Hardy-Weinberg equilibrium were removed from the analyses. GeneAlEx 6.5 (Peakall and Smouse 2012) was used to describe allele frequencies, number of alleles (Na), effective number of alleles (Ne), observed (Ho) and expected (He) heterozygosity, Shannon's information index (I) and the ability in distinguishing individuals through the probability of identity test (PID). To overcome the bias caused by the presence of closely-related individuals in the population, which are more likely to share identical genotypes by chance (Waits et al. 2001), the equivalent probability for pairs of siblings (PIDsibs) was also calculated. These statistical tests were computed on SNP and STR-reference data to compare the genetic variability described by different marker datasets.

Parentage analyses

The reliability of parentage analysis is maximised when all individuals of the family trio (dam, sire and offspring) are sampled and fully genotyped. However, poor and degraded DNA can lead to incomplete or missing genotypes. The development of a protocol preserving the maximum number of individuals while minimising the effect of incomplete genotypes is advantageous. To test for the incidence of these limiting factors, two sample datasets were created, the first including samples with a SNP call rate ≥ 70% and a second with a reduced number of individuals, only those with a SNP call rate ≥ 90%. Parentage relationships were evaluated using FRANz v.2 (Riester et al. 2009), a commonly used likelihood-based parentage assignment software that incorporates multi-generation analyses using polymorphic co-dominant markers. Values of mistyping error rate and the maximum number of mismatches were set at 0.02 and 1, respectively.

Moreover, kinship analysis was also tested in Colony v. 2.0.6.4 (Jones and Wang 2010) to verify the robustness of results obtained from FRANz. A subset of genotypes was created for each monitoring year (n = 13), avoiding multi-generation analyses. Each annual parentage analysis includes bears born in the year of reference and putative parents. Year of birth and reproductive age (3 years for females and 5 years for males) were evaluated, based on the long-term monitoring programme (i.e. direct observations of females with cubs monitored through radio- and GPS-telemetry, at hair-trap sites equipped with camera traps and through *a posteriori* genetic parental assignments). We anticipate that reproductive traits (i.e. age of primiparity, interbirth interval, mean litter size) obtained from genetic data are always concordant with the reproductive biology of the species (Giangregorio 2018). Accepting these assumptions, we removed all bears who died within the last year, as they could not have taken part in the reproductive event in the year of reference.

Parameters used in FRANz v.2 and Colony v. 2.0.6.4 software, have been set following the procedure described in Davoli et al. (2018) and reported in Suppl. material 1: table S5.

To test the reliability of parental assignments, family trios obtained with SNPs were compared with those obtained formerly with STR. This was done by calculating the number of congruent, missing and incongruent parentage assignments in addition to significance values in the detected family trios. Missing assignments caused by the lack of the true parents in the SNP dataset were also deemed "congruent". Finally, we combined SNP and STR genotypes in a single dataset and compared the results obtained through SNPs and STRs alone.

Results

Genotypes and marker datasets

Out of the 51 autosomal SNPs analysed, eight did not amplify, 15 were monomorphic, 11 showed unclear cluster affiliation or unusual clustering patterns, 5 showed call rates ≤ 70% and one showed a departure from the Hardy Weinberg equilibrium and were subsequently removed from the analyses. A total of 45 SNPs was thus retained for further analysis. Out of the 74 genotyped individuals, 54 were retained (including five founders and three bears from the remnant Dinaric population) as they had SNP genotyping call rates ≥ 70% (mean call rate was 85%), while 20 were rejected. Out of these 54 bears, 41 (including three founders and two Dinaric bears) showed a percentage of call rate ≥ 90% (mean call rate 97%). Details on genotyping success are shown in Suppl. material 1: table S4. A flowchart showing the filtering process with results is shown in Suppl. material 1: table S3.

The genotyping of 45 SNPs correctly identified 54 individual bears and sex determination, based on six SNPs on the sex chromosomes, confirmed STR-based results in 42 out of 54 cases (77%). The remaining 12 cases did not show incongruent results, but partially missing data at SNPs on the sex chromosomes prevented the sex determination. Amongst the 54 samples, 12 were replicated four times and showed 92% positive PCR amplifications amongst loci and 92% amongst samples. Allelic dropout interested only 1.6% of loci (mean value = 0.04) and 1.4% of samples (mean value = 0.02). Amongst 45 SNPs, 27 showed the three genotypic representatives (e.g. AT/TT/AA). The following statistics on variability and parentage analyses were performed using the reference dataset of 15 STRs, the total amount of 45 SNPs and the reduced dataset of 27 most variable SNPs. In addition, datasets formed by the combination of 15 STRs with 27 SNPs and 15 STRs with 45 SNPs were also processed.

STR and SNP marker statistics

Summary statistics for single markers are shown in detail in Suppl. material 1: table S6, while average statistics data of all marker subsets are summarised in Table 1. The mean number of alleles per locus (Na) was 4.53 (SD = 0.291) using the reference STRs panel, while 2.90 (SD = 0.215) and 2.63 (SD = 0.159) Na were recorded using the two STR and SNP combined panels. PID values ranged from 1.01×10^{-05} using 27 SNPs to 8.52×10^{-13} using the combined panel of 45 SNPs and 15 STRs, while PIDsibs values ranged from 2.17×10^{-10} to 1.95×10^{-26}. Values are plotted in Fig. 2.

Table 1. Marker summary statistics. The mean number of loci typed in 51 brown bear samples from the Central Italian Alps. (N), the mean number of alleles per locus (Na), the mean effective number of alleles (Ne), Shannon's information index (I), observed (Ho) and expected (He) heterozygosity. Standard error values (SE) are in brackets.

MARKER SETS	N (SE)	Na (SE)	Ne (SE)	I (SE)	Ho (SE)	He (SE)
15 STRs	51.00 (0.000)	4.53 (0.291)	3.25 (0.223)	1.25 (0.084)	0.73 (0.045)	0.66 (0.040)
27 SNPs	47.30 (0.443)	2.00 (0.000)	1.74 (0.049)	0.59 (0.023)	0.43 (0.026)	0.41 (0.020)
45 SNPs	47.18 (0.405)	2.00 (0.000)	1.58 (0.044)	0.52 (0.022)	0.38 (0.022)	0.34 (0.019)
27 SNPs & 15 STRs	48.26 (0.348)	2.90 (0.215)	2.28 (0.141)	0.83 (0.059)	0.54 (0.032)	0.50 (0.026)
45 SNPs & 15 STRs	47.88 (0.342)	2.63 (0.159)	2.00 (0.114)	0.70 (0.049)	0.47 (0.028)	0.42 (0.025)

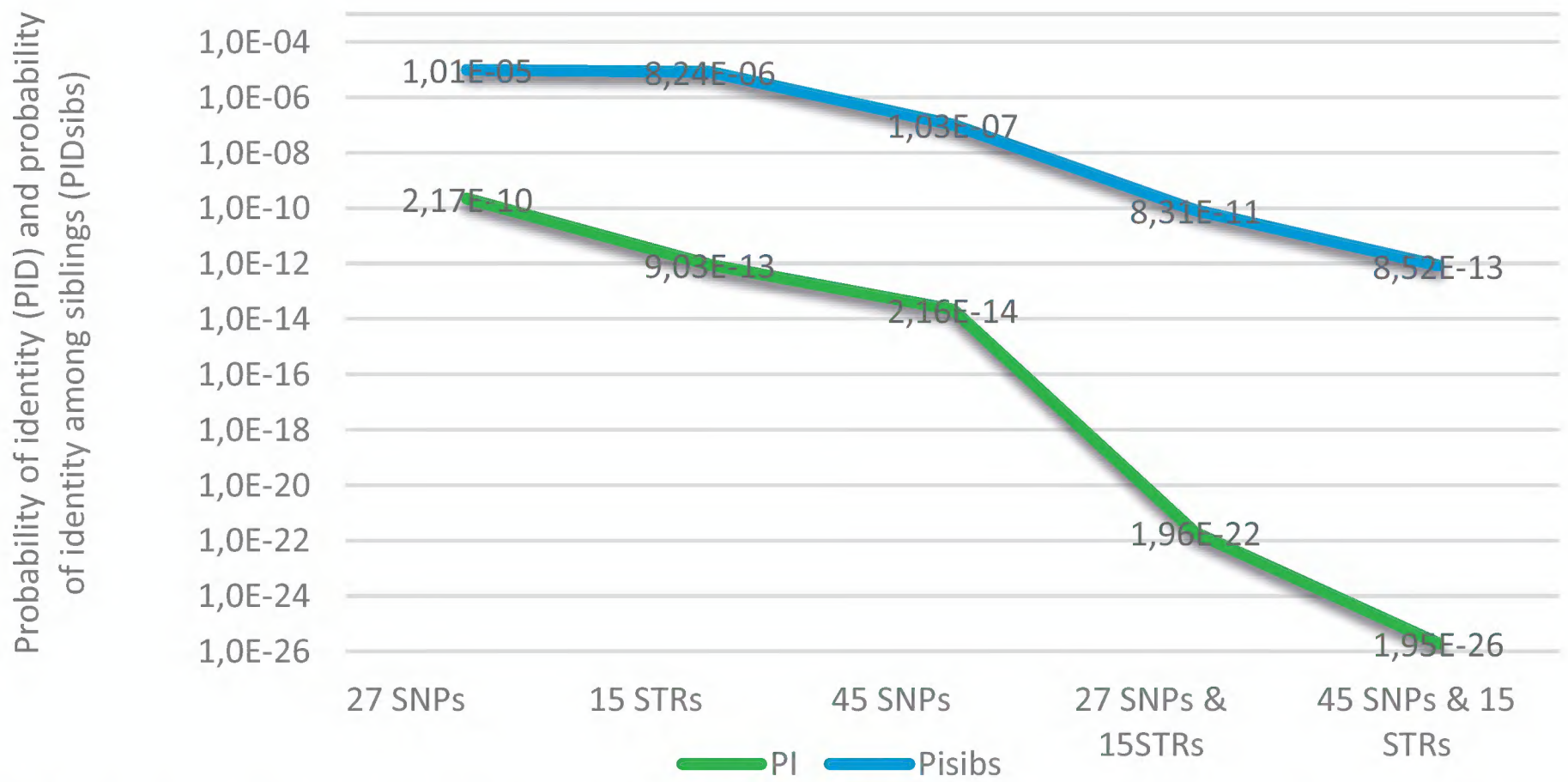

Figure 2. PID and PIDsibs values for increasing locus combination in the brown bear Alpine population. The values are calculated for the five marker sets: 27 most variable SNPs (showed the three genotypic representatives), the reference dataset of 15 STRs, the total amount of 45 SNPs, the combination of 15 STRs with 27 SNPs and the combination of 15 STRs with 45 SNPs.

Parentage analyses

Parentage tests were performed in two independent analyses excluding the five founder individuals in a total of individuals with call rates ≥ 70% (n = 49, 100%) and individuals with call rates ≥ 90% (n = 38, 77.5%). Amongst bears with call rates ≥ 70% and ≥ 90%, 2 out of 21 (9.5%) and 7 out of 17 (41.1%) of true parents (detected using the STR-based reference data) were removed from the dataset because of the filtering process, respectively. As a result, three (3.2%) and 30 (27.7%) assignments with the true parent were impossible to detect in the two analyses.

Parental assignments using bears with a call rate ≥ 70% and the complete set of 45 SNPs resulted in 8.16% of missing assignments and 7.14% inconsistencies with the STR reference data. Similar results were obtained using the reduced panel of 27 SNPs resulting in 11.22% of missing assignments and 6.12% of inconsistencies. This slight difference is probably due to the greater reliability of the 27 SNPs compared to the 45 SNPs as the former are characterised by

the presence of all the three allelic forms. Variation was detected in samples with call rates ≥ 90%: the percentage of inconsistencies is similar (7.89% using 45 SNPs and 9.21% using 27 SNPs), but no missing assignments were found.

Using the two combinations of 15 STRs and 45/27 SNPs, amongst bears with call rates ≥ 70%, 8.16% and 6.12% had missing assignments and no inconsistencies were found. All parental assignments identified using bears with a call rate ≥ 90% were concordant with the STR reference data, despite the absence of a high proportion of parents in the dataset. On bears with SNP call rate ≥ 70%, the 15 STRs showed congruent results on all family trios, while bears with SNP call rate ≥ 90% led to one missing assignment (1.30%) and one incongruent assignment (1.30%). Proportions of congruent, incongruent and missing sire/dam assignments are summarised in (Table 2).

As expected, no parents were found for the three bears of Dinaric origin and all parental assignments were confirmed using Colony 2.0.6.4. Amongst congruent assignments, no mismatches were found and the mean number of common loci typed in the family trios was 58.7 out of 60 (min = 56, max = 60). Individual details about the combination of SNP and STR markers individuals' assignment are reported in Suppl. material 1: table S7.

Parental assignment probabilities were calculated for bears with the 36 most reliable genotypes (the Dinaric bears being excluded) using the five marker subsets. Results are displayed in Fig. 3: 27 SNPs showed the lowest mean values (mean = 0.75; min = 0.26), followed by 45 SNPs (mean = 0.84; min = 0.12). In both cases, a high number of bears with a parental assignment probability low-

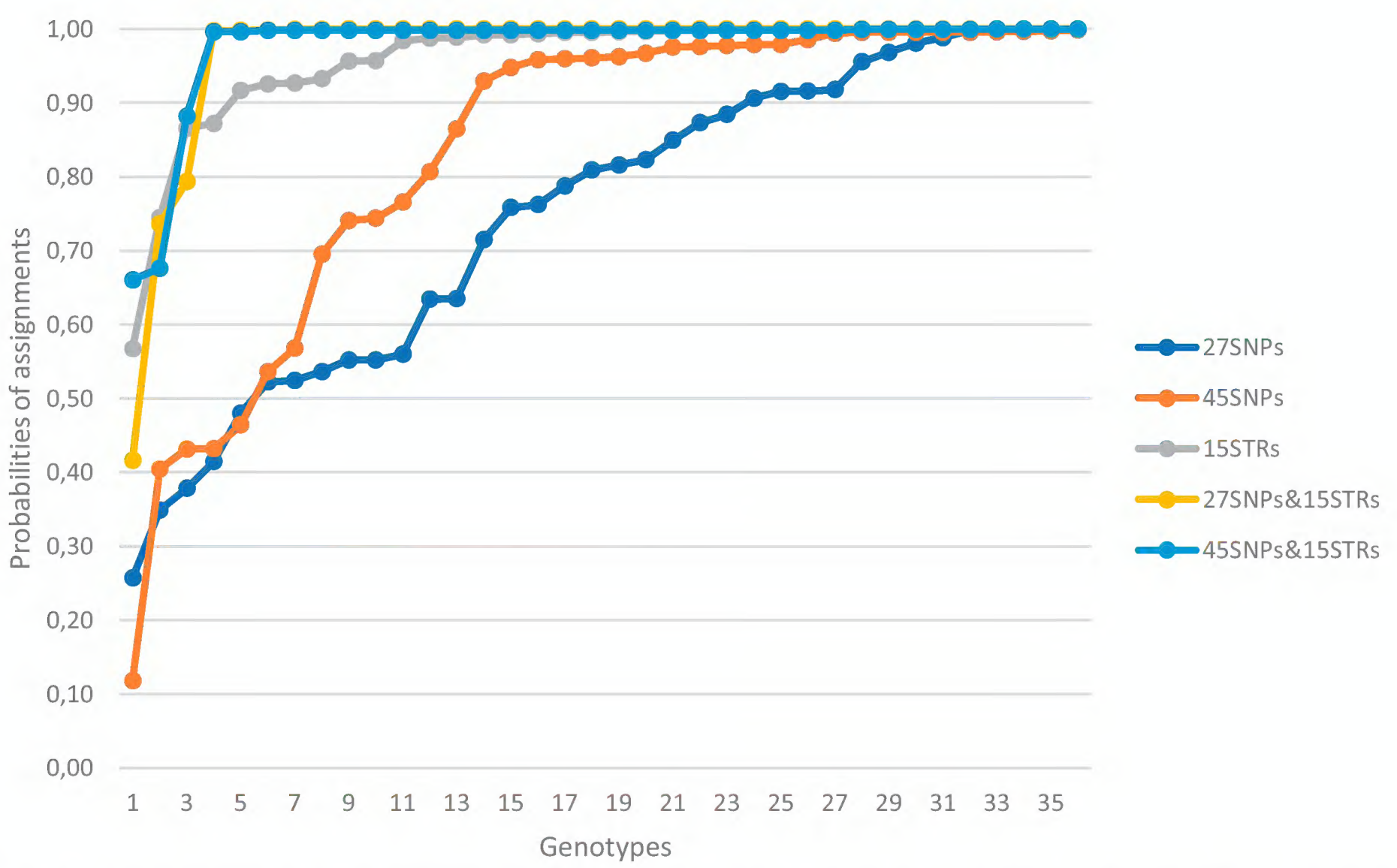

Figure 3. Parental assignment probabilities using FRANz v.2. The probabilities are calculated for the 36 most reliable bear genotypes born in the Central Italian Alps (the three bears of Dinaric origin are excluded) using the five marker sets described in Table 1. Probabilities are shown in ascending order for each genotype. Each marker set is indicated with a different colour (see the legend).

Table 2. Results of parental assignments using FRANz v.2. Values and percentages of correct, incongruent and missing parental assignments of 49 bear genotypes with call rate ≥ 70% (a) and 38 bear genotypes with call rate ≥ 90% (b) in the Italian Alpine brown bear population. Results are reported for each subset of SNP/STR marker. The total number of assignments to be determined for 49 (n = 98) and 38 (n = 76) bears genotypes are shown on the bottom row.

Call rates ≥ 70%	**27SNPs**	**45SNPs**	**15STRs**	**27SNPs&15STRs**	**45SNPs&15STRs**
Congruent	81 (82.65%)	83 (84.69%)	98 (100%)	92 (93.87%)	90 (91.8%)
Not assigned	11 (11.22%)	8 (8.16%)	0	6 (6.12%)	8 (8.16%)
Incongruent	6 (6.12%)	7 (7.14%)	0	0	0
TOT n assignments	**98**	**98**	**98**	**98**	**98**
Call rates ≥ 90%	**27SNPs**	**45SNPs**	**15STRs**	**27SNPs&15STRs**	**45SNPs&15STRs**
Congruent	69 (90.78%)	70 (92.10%)	74 (97.36%)	76 (100%)	76 (100%)
Not assigned	0	0	1 (1.31%)	0	0
Incongruent	7 (9.21%)	6 (7.89%)	1 (1.31%)	0	0
TOT n assignments	**76**	**76**	**76**	**76**	**76**

er than one was detected (31 and 27 out of 36 using 27 or 45 SNPs, respectively). For both STR/SNP marker combinations, mean and minimum probabilities of parental assignments were much higher (0.97/0.98; min = 0.42/0.66) and the number of bears with a probability of parental assignments lower than one was retrieved only in three cases. The probabilities of parental assignment using 15 STR markers showed intermediate mean values (0.96; min = 0.57).

Discussion

Our study demonstrated that a combination of SNPs and STRs provided robust assessments of parentage in the Italian Alpine brown bear population and performed better than STRs when a high proportion of true parents was not present in the dataset. The absence of parents in the dataset simulates a common situation in long-term monitoring projects of expanding populations, in which not all individuals are usually sampled. Interestingly, the numbers of assignments congruent with the reference STR-based data and probability values do not differ significantly when using the reduced set of the most variable 27 SNPs (He = 0.43) or the complete set of 45 SNPs (He = 0.38).

This result indicates that 27 SNPs, in combination with 15 STRs, are sufficient to considerably enhance data reliability compared to the use of 15 STRs alone. Combinations of SNPs and STRs were found to be more efficient than a higher number of SNPs alone and also in other species as in the African penguin (*Spheniscus demersus*; Labuschagne et al. (2017)). These authors obtained a > 99% correct cumulative parentage assignment probability, comparable to that obtained in this study. A similar study showed that a combined dataset of 33 SNPs and 6 STRs was most informative, with the highest confidence level, in the white rhino (*Ceratotherium simum*; Labuschagne et al. (2017)).

Conversely, 45 SNPs were not sufficient to determine parentage relationships in the Alpine population, although a similar number of SNPs were found to be adequate for assigning parents by Kaiser et al. (2017). They found 40 SNPs with a Ho value (0.37), similar to that obtained with 45 SNPs in our study (0.38), to be effective in the socially monogamous black-throated blue warbler (*Setophaga caerulescens*) and claimed that this small number of SNPs were

just as powerful as six multi-allelic STRs. However, the mother was known, thus only a paternity test had to be performed. Additionally, Tokarska et al. (2009) and Labuschagne et al. (2015) found that SNPs performed slightly better than STRs in an ex-situ African penguin and in the European bison (*Bison bonasus*) population, respectively. However, differently from this study, invasive samples were used in all these former investigations.

Due to the wide-roaming of young male bears, international cooperation amongst labs involved in the monitoring of the species is pivotal for the conservation of the species in the Alps.

The long-term and intense monitoring of the brown bear population in the Italian Alps allowed us to empirically evaluate the performance of SNP markers in a wild population using non-invasive samples to assess family relationships. This information is usually difficult to achieve because field data (such as telemetry and direct observations of females with cubs), multiple sampling of individuals and multiple amplification of STR loci over years, are rarely available to confirm parentage assignments. In this study, reproductive data obtained from genetic and field data were available from more than a decade of research and management efforts and were always concordant with the reproductive biology of the species (see Giangregorio et al. (2018) for further information about the demographic history of the population). By considering the results obtained from this study, we can state that the 15 reference STRs and 27 SNPs can assess parental pairs, also with reduced genetic diversity. In the future, these markers could also be applied to other populations to make data comparable and useful for species conservation and management.

Genetic markers may also be less variable and informative when applied to a different population than the one for which they were developed; these SNPs were selected for being informative in the Scandinavian population and, as expected, only a portion was highly variable (51/96 = 53%). Ascertainment bias, due to the SNPs being selected for the Scandinavian bear population, likely contributed to the lower power of parentage assignment. Some of the 45 SNPs used in this study had low minor allele frequencies for the Alpine population, lowering their discretionary power.

In addition, a substantial number of studies concluded that SNP markers are entirely appropriate for parentage analyses, but the empirical data, thus far, indicate that a suite of 100–200 SNPs is generally needed to provide resolving power equal to or better than that provided by the available STR markers for the species under consideration (Flanagan and Jones 2019).

The integration of an additional set of SNPs specifically developed for the Alpine population would likely improve the effectiveness of parentage analyses, solving the problem of the low number of variable SNPs identified in this study. Benazzo et al. (2017) recently sequenced the brown bear genome and found several SNPs in the Apennine brown bear population that might prove variable in the Alpine population. SNP discovery within these variable regions could be conducted through advanced methodologies, such as genotyping-by-sequencing or RAD-tag sequencing (Gutierrez et al. 2017; Andrews et al. 2018; Zhao et al. 2018).

In this study, when using a lower call rate threshold (≥ 70%), a few incongruent parental assignments were found and a few assignments were missed. These errors are probably due to a combination of two factors: the lower number of common loci typed in the correct family trio and a few genotyping errors

amongst SNPs. Our SNP results highlighted the importance of using special precautions when working with non-invasive samples, such as pre-selecting samples with high call rates (≥ 90%). Additionally, Kaiser et al. (2017) demonstrated that missing data in the SNP genotypes decrease the rate of congruent parental assignments. As in our study, increasing the filtering threshold of sample call rate by 70% and 90% reduced the number of offspring typed, but increased concordance in parentage assignments between STRs and SNPs. Missing data resulted in incomplete genotypes, which affected the ability of SNPs to resolve parentage in a few cases. The importance of using samples with a high call rate and including replicates when working with SNPs and non-invasive samples was also discussed by von Thaden et al. (2017). Our results thus emphasise the necessity of developing strategies and protocols for the use of SNPs when working with poor-quality samples, such as non-invasively collected faeces or hair.

We also underline that SNPs have some intrinsic disadvantages, especially when using non-invasive samples: since SNPs are bi-allelic, it is not straightforward how to recognise samples containing DNA from multiple individuals and rules concerning the number of replicates and call rates needed to obtain reliable genotypes are lacking.

More SNPs are needed to perform parentage analysis with an information content comparable or superior to that obtained through STRs (Morin et al. 2004) but, even with low error rates, parentage analysis may become problematic as the number of loci screened becomes very large (Hauser et al. 2011; Christie et al. 2013), thus making it necessary to find a compromise between the number and type of markers.

Despite the difficulties that may manifest when working with non-invasive samples and given the mentioned ascertainment bias, our results showed that a combination of 27 SNPs and 15 STRs was an effective panel in identifying parentage relationships in an isolated brown bear population, although several half- or full siblings amongst putative parents are present. The use of SNPs in parentage analysis is thus promising even if it should be evidenced that multiple factors could contribute to jeopardise the reliability of the results.

Considering the data obtained in this study, we provided simple guidelines to perform efficient parentage analysis in wild populations using non-invasive samples with STRs and SNPs: a) amplifying a congruent number of STRs and determining sex through the amplification of sex-specific regions for all collected samples should be the first step. The STR amplification can be used to discard bad-quality samples and identify single individuals; b) amongst multiple samples of the same individual, the one with the lower genotyping error rate and higher positive amplifications with STRs may be chosen for SNP genotyping; c) a SNP genetic data bank, including all putative parents, can be developed. 96 × 96, 48 × 48 or 192 × 24 plates can be used on the Fluidigm Biomark Platform, depending on the number of individuals and SNP availability; d) parentage analysis can be performed using FRANz, combining an appropriate number of SNP and STR markers, to allow for multi-generational analysis.

However, our data highlighted the need of using good-quality samples (e.g. call rates ≥ 90%, given our results) with a low likelihood of allelic drop-out. Indeed, parental assignments are particularly vulnerable to genotyping problems, as parent-offspring pairs must share at least one identical allele at each locus (Wang 2019). Eriksson et al. (2020) outlined a detailed protocol for cost-effec-

tive and accurate non-invasive SNP genotyping optimised for degraded DNA, while Ekblom et al. (2021) were the first to successfully reconstruct the pedigree in a wild population of wolverine (*Gulo gulo*) using SNP-genotyping of non-invasive samples, with individuals known to have high levels of relatedness (e.g. full siblings from inbred mating). In addition, choosing the most informative SNPs whether pursuing a SNP-PCR, SNP-chip or targeted capture method, is imperative, as concluded also by Flanagan and Jones (2019). Lastly, due to the risk of ascertainment bias, the SNP panel should be developed and/or validated for the population of interest, since it cannot always be easily transferable to other parts of the distribution range (Morin et al. 2004; Ekblom et al. 2021). Moreover, the use of STRs or SNPs of both of them can be considered depending on the situation for sample quality and quantity.

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Additional information

Conflict of interest

The authors have declared that no competing interests exist.

Ethical statement

No ethical statement was reported.

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Author contributions

Conceptualization: FD, GS, PG, NM. Data curation: GS, LP, PM, SF, PG, FD. Formal analysis: PG, GS. Investigation: NM, AJN, FD. Methodology: AJN, NM, GS. Project administration: FD, NM. Resources: LP, GS, SF, PM, AJN. Supervision: FD, GS, NM. Validation: NM, AJN, FD, GS. Writing - original draft: PG. Writing - review and editing: PM, LP, NM, SF, AJN, GS, FD.

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Data availability

The data that supports the findings of this study are available in the supplementary material of this article.

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Supplementary material 1

Genetic and field data

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Data type: genetic and field data (word document)

Explanation note: Sample information, parentage relationships, marker summary statistics, FRANz v. 2 and Colony v. 2.0.6.4 output data.

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